A3 adenosine receptor agonist potentiates natural killer cell activity

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Abstract. Activation of the Gi-protein-coupled A3 adenosine receptor (A3AR) has been reported to be involved in the inhibition of tumor cell growth. A3AR is highly expressed in tumor cells whereas lower expression was noted in a variety of normal cells. Recently we showed that A3AR activation in melanoma cells resulted in growth inhibition via a direct anti-proliferative effect which entailed cell cycle arrest in the G_0/G_1 and down-regulation of cyclin D1 and c-Myc. In the present study we present an additional mechanism demonstrating that A3AR agonists activate natural killer (NK) cells which further enhance the anti-tumor effect of this group of molecules. NK cells mediate the natural cytotoxicity and their number and function is reduced in cancer patients. We show Cl-IB-MECA to inhibit tumor development via the activation of NK cells is an additional mechanism which accounts for the anti-tumor effect of A3AR agonists. This effect was noted at a low dose of 10 μg/kg, demonstrating that the response is exclusively A3AR mediated. Treatment of naïve mice for four days yielded the highest effect on NK cell potentiation. In mice inoculated with B16-F10 melanoma cells and treated each orally with Cl-IB-MECA, melanoma growth inhibition correlated with higher serum level of IL-12 and potentiation of NK cells. Moreover, in adoptive transfer experiments in melanoma bearing mice, marked inhibition of lung metastatic foci was noted upon engraftment with splenocytes derived from Cl-IB-MECA treated mice. Taken together, the ability of Cl-IB-MECA to inhibit tumor development via the activation of NK cells is an additional mechanism which accounts for the anti-tumor effect of A3AR agonists.

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Introduction

Strategies to treat human cancers through manipulation of the anti-tumor immune response have generated considerable interest over the past two decades. NK cells constitute 5-15% of circulating lymphocytes, and they have a similar frequency in the spleen (1). NK cells mediate the natural cytotoxicity process and are the major components of the immune surveillance mechanism, where they recognize and kill both infected and transformed cells. NK cells utilize several mechanisms to lyse target cells including calcium-dependent release of granules containing perforin (2), release of granzymes A and B (3) and the induction of apoptosis through a family of death receptors (4,5).

A role for NK cells in tumor surveillance has been postulated based on the finding that NK cells are deficient in patients with solid tumors and leukemia (6). Clinical observations have indicated that natural cytotoxicity is reduced in cancer patients, possibly due to tumor volume or dissemination. Furthermore, anti-cancer treatment (e.g., surgery, hormonal modulation, radiotherapy and chemotherapy) can also result in suppression of natural cytotoxicity (7). The activity of NK cells from patients with malignant melanoma was found to be decreased relative to normal controls. This abnormality was significantly correlated with advancing stage of disease (8). IL-12, previously designated NK cell stimulatory factor (NKSF), mediates several biological activities of NK cells and is thus considered to have anti-cancer effect (9,10).

The A3AR is one out of four cell membrane receptors which bind adenosine and are classified as A1, A2A, A2B and A3 (11,12). Activation of A3AR, a Gi-protein cell surface receptor, induces inhibition of adenylyl cyclase activity and cAMP formation leading to a decreased level of the effector PKA. Low receptor expression is a characteristic of most normal tissues while tumor cells show high expression which suggest this receptor as a target for the induction of tumor growth arrest (13-15).

The synthetic A3AR agonists IB-MECA and Cl-IB-MECA, at low concentrations (0.01-10 μM), were found to have an inhibitory effect on melanoma cell growth *in vitro* (16). The mechanism was found to involve inhibition of telomerase

activity and cell cycle arrest in the G_0/G_1 phase leading to a cytostatic effect (17,18). Additionally, it was found that agonists to A3AR cause tumor growth inhibition by de-regulating the Wnt signaling pathway resulting in down-regulation of the cell cycle genes cyclin D1 and c-Myc. The A3AR antagonist, MRS 1523, reversed the inhibition of cell growth, counteracting Cl-IB-MECA and IB-MECA's effect, demonstrating that the response is A3AR mediated (19).

In a syngeneic mice model (B16-F10 melanoma in C57Bl/6J mice) IB-MECA or Cl-IB-MECA inhibited tumor growth when administered orally at low dosages (3-100 μg/kg) (Neoplasia, Exp Cell Res, anti-cancer drugs). Interestingly, the tumor inhibitory effect was of higher magnitude *in vivo* in comparison to that seen *in vitro*. This observation led us to assume that in addition to the direct anti-cancer effect of A3AR agonists, they may act as immunomodulators and activate NK cells.

In the present study we show that Cl-IB-MECA potentiates the activity of NK cells in naïve and tumor-bearing mice via the induction of IL-12 production. This was demonstrated by direct assessment of NK cell activity *ex vivo* and by adoptive transfer studies which proved the efficacy of the potentiated NK cells *in vivo*.

Materials and methods

Tumor cells. B16-F10 melanoma cells were purchased from the American Type Tissue Culture Collection (ATCC, Rockville, MD). Cells were maintained in RPMI medium supplemented with 10% FBS, 200 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were transferred to a freshly prepared medium twice weekly.

Drugs. The A3 adenosine receptor agonist 2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-N-methyl-uronamide (Cl-IB-MECA) was purchased from RBI, Sigma, USA. The compound was dissolved in dimethylsulfoxide and then dilutions were obtained in PBS for the *in vivo* experiments.

Ex vivo NK activity. To test the activity of NK cells in Cl-IB-MECA treated mice, splenocytes were separated from mice previously treated with the drug. *Ex vivo* NK cell activity was tested by a standard 4 h [^{51}Cr]-release assay, using YAC lymphoma cells as targets. Splenocytes ($1x10^6$) were cultured in 96-well plates and resuspended in RPMI containing 10% FBS. The YAC lymphoma cells were labeled with 100 μci of $Na_2[^{51}Cr]O_4$ at 37˚C for 1 h. Cells ($2x10^4$) were then resuspended and mixed with the effector cells at the E:T ratio of 50:1, in a volume of 200 μl. After 4 h of incubation at 37˚C in 5% CO_2, plates were centrifuged and supernatants were counted in a γ-counter (LKB).

NK cytotoxicity was calculated using the following equation:

$$\% \text{ lysis} = \frac{\text{cpm experiment - cpm spontaneous}}{\text{cpm maximal - cpm spontaneous}} \text{ x100}$$

where spontaneous and maximal counts per minute (cpm) were determined by measuring supernatants' cpm of target cells (alone or in the presence of 1% SDS). The spontaneous release was below 10% of the maximal release throughout the experiments.

In vivo studies. Standardized pelleted diet and tap water were supplied for the mice used for these studies. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Rabin Medical Center, Petah Tikva, Israel.

ICR male mice (Harlan Laboratories, Jerusalem, Israel) aged 2 months, weighing an average of 20 g were used for the *in vivo* activation of NK cells. Cl-IB-MECA was administered orally once daily for two consecutive days, and spleens were withdrawn 48 h after Cl-IB-MECA's second administration for *ex vivo* NK activity assay. Serum samples were collected for evaluation of IL-12 levels. Each group contained 5 mice and the study was repeated 4 times.

C57BL/6J, male mice (Harlan Laboratories, Jerusalem, Israel) aged 2 months, weighing an average of 20 g were used for the artificial melanoma lung metastasis model. B16-F10 melanoma cells ($2.5x10^5$) were inoculated into mice intravenously (i.v.). The control group was treated once daily with the vehicle only, administered orally. Cl-IB-MECA at a dose of 10 μg/kg body weight was administered orally once daily, starting one day after tumor cells inoculation. Mice were sacrificed after 15 days, lungs removed and black metastatic foci were counted using a dissecting microscope. Spleens withdrawn from the mice were tested for *ex vivo* NK activity. Serum samples were collected for evaluation of IL-12 levels. Each group contained 15 mice and the study was repeated 3 times.

Adoptive transfer experiments. Spleens were removed from the B16-F10 melanoma bearing mice which were previously treated orally daily with 10 μg/kg Cl-IB-MECA (as described above). For controls, splenocytes were derived from melanoma-bearing mice treated with the vehicle only. The splenocytes ($5x10^6$) were injected i.v. to two groups of mice that were inoculated with $2.5x10^5$ B16-F10 melanoma cells 4 days earlier. Mice were sacrificed after 15 days, lungs removed and the black metastatic foci were counted using a dissecting microscope. Each group contained 10 mice and the study was repeated 3 times.

IL-12 analysis in serum sapmles. Serum samples were collected and kept at -70˚C till assayed. IL-12 serum level was assessed utilizing commercial murine ELISA kit of R&D systems, Minneapolis, MN.

Statistical analysis. The results were statistically evaluated using the Student's t-test. For statistical analysis, comparison between the mean value of different experiments was carried out. The criterion for statistical significance was $p<0.05$.

Results

To test the effect of Cl-IB-MECA on NK cell activity *in vivo*, mice were orally treated with different dosages of Cl-IB-MECA (10, 100 and 1000 μg/kg body weight). Splenocytes were derived from mice and tested for NK activity utilizing the [^{51}Cr]-release assay (Fig. 1). Marked increase in NK activity

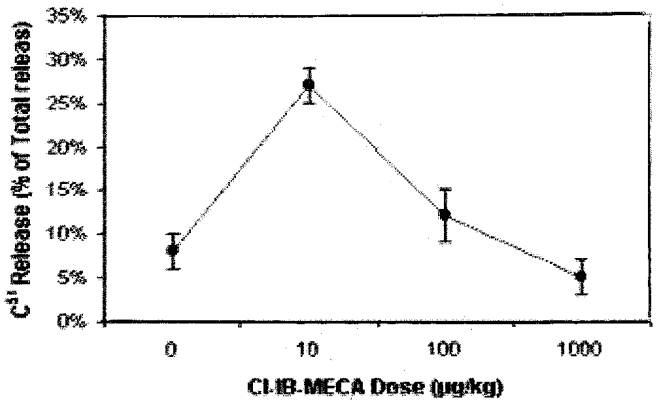

Figure 1. Cl-IB-MECA induces a stimulatory effect on NK cells activity. Cl-IB-MECA was administered orally to mice at various dosages (10-1000 µg/kg body weight). Maximal activity of Cl-IB-MECA was observed at a dose of 10 µg/kg body weight.

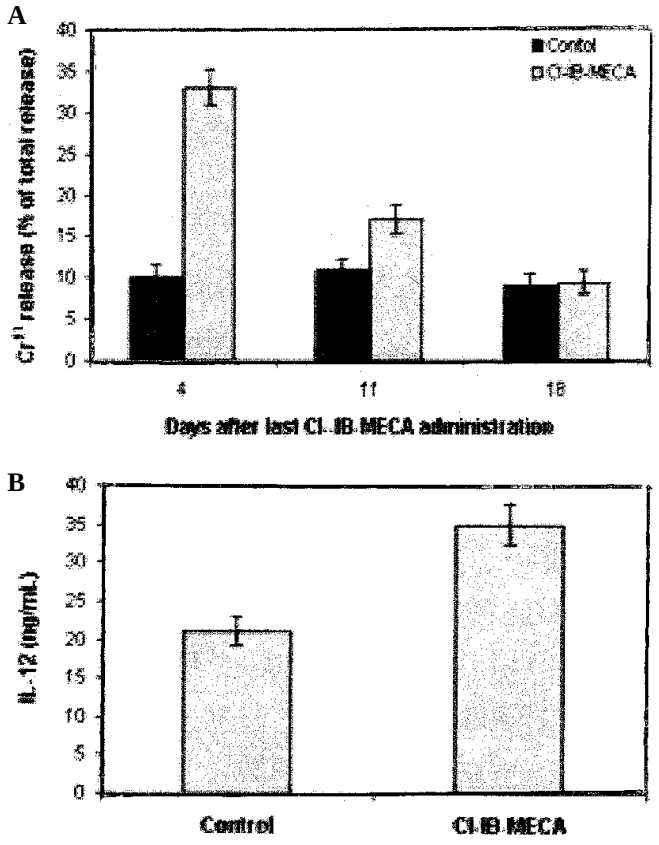

Figure 2. Cl-IB-MECA induces time kinetic response on NK cell activity. Mice were treated orally for 2 consecutive days with Cl-IB-MECA (10 µg/kg) (A). Serum IL-12 level in the CL-IB-MECA treated mice was evaluated by ELISA (B).

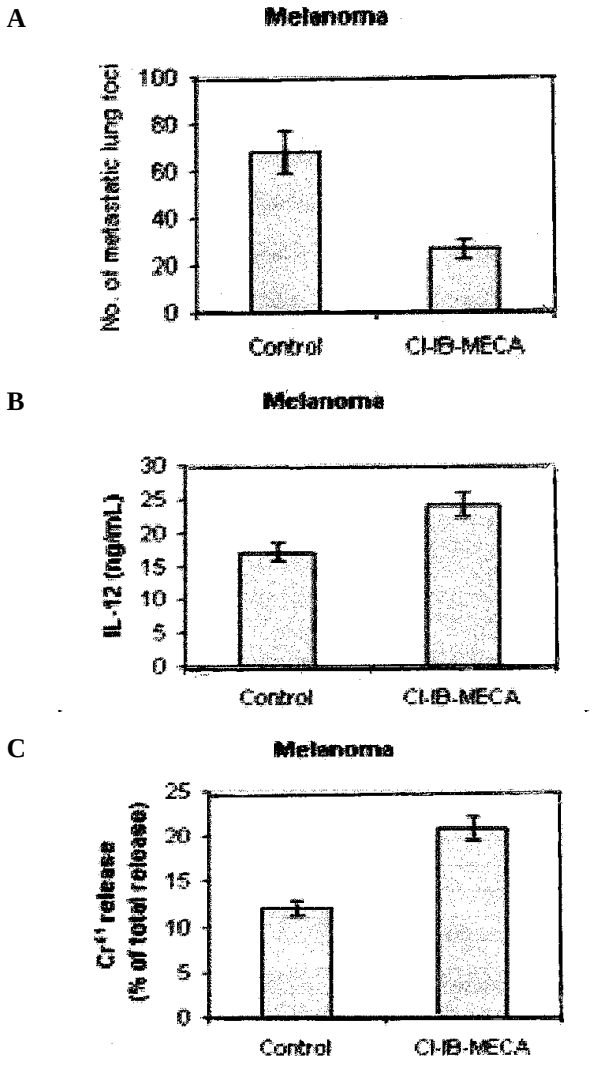

Figure 3. Cl-IB-MECA inhibits the development of melanoma. Cl-IB-MECA inhibited the development of lung metastatic foci (A). Increased serum levels of IL-12 was observed in Cl-IB-MECA treated mice (B). An increased NK cell activity was exhibited in solenocytes derived from Cl-IB-MECA treated melanoma bearing mice in comparison to control (C).

was noted at 10 µg/kg, however at 100 µg/kg a slight response was noted while no response was observed on 1000 µg/kg. To examine the time kinetic effect of Cl-IB-MECA (10 µg/kg) on NK activity, mice were treated for 2 consecutive days and after 4, 8 and 11 days splenocytes were derived and tested for NK activity. The results show a time-dependent response, with high NK cell activity on day 4, reaching the control level on day 11 (Fig. 2A). An increase in the level of the cytokine IL-12 was noted in serum samples derived from Cl-IB-MECA treated mice (Fig. 2B).

Inhibition of lung metastatic foci development in mice inoculated with B16-F10 melanoma cells was observed upon daily treatment with Cl-IB-MECA (Fig. 3A). In these mice, serum IL-12 level was 41% higher than that of the control group (Fig. 3B). Moreover, an increase in NK cell activity was seen in spleens derived from Cl-IB-MECA treated melanoma-bearing mice (Fig. 3C).

To further assess whether Cl-IB-MECA induces potentiation of NK cells, adoptive transfer experiments were performed. Splenocytes from melanoma-bearing mice (on day 15 following tumor inoculation and daily treatment with Cl-IB-MECA) were engrafted to mice which were inoculated 4 days earlier with B16-F10 melanoma cells. The mice were sacrificed on day 15 and metastatic lung foci were evaluated. A marked inhibition of melanoma development was observed in the group engrafted with splenocytes derived from Cl-IB-

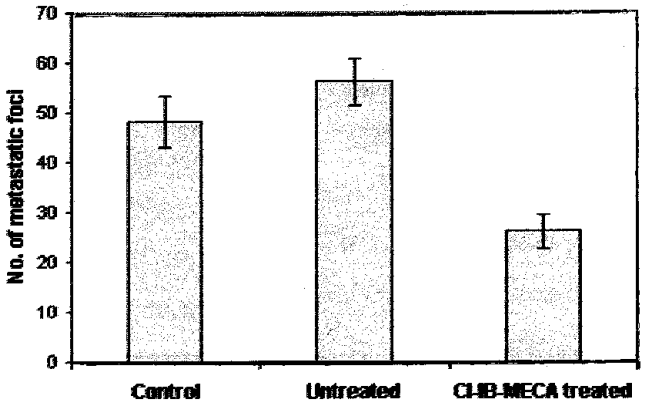

Figure 4. Adoptive transfer experiment. Splenocytes derived from Cl-IB-MECA treated mice engrafted of to melanoma-bearing mice.

MECA treated mice, demonstrating the capability of Cl-IB-MECA to induce NK cell activation (Fig. 4).

Discussion

This study presents data showing that the A3AR agonist, Cl-IB-MECA, induces IL-12 production and NK cell activation upon its oral administration to naïve or tumor-bearing mice.

Although NK cells are capable of killing several tumor types *in vitro*, progressive functional defects in NK cell proliferation and cytolytic activity were recorded in cancer patients (20). Possible explanation for the decreased NK function was attributed to the presence of purine metabolites in the tumor microenvironment, known to be released from lysed cells and capable of inhibiting NK activity. Adenosine, was found to exert similar suppressive effect on NK activity by giving rise to increased adenylyl cyclase activity and cAMP production. Elevation of intracellular cAMP results in impairmed function and proliferation of NK cells (21). The mechanism underlying the inhibitory effect of cAMP on NK activity includes decreased capacity of the effector cells to conjugate to target cells and impairment of the lytic efficiency of the NK cells (22-24). Thus, agents capable of down-regulating cAMP activity may act as potent stimulators of NK cells.

A3AR belongs to the family of the Gi-protein associated cell surface receptors. High receptor expression was found in different tumor cell lines, including Jurkat T, pineal gland, breast cancer and melanoma cells. Receptor activation leads to its internalization and the subsequent inhibition of adenylyl cyclase activity, cAMP formation and protein kinase A (PKA) expression, resulting in the initiation of various signaling pathways (25).

It is thus reasonable that in the present study, activation of the A3AR potentiated NK cell activity via the inhibition of cAMP production. Interestingly, stimulation of NK cell activity was noted only at a dose of 10 μg/kg. Based on the affinity profile of Cl-IB-MECA, at this concentration it activates exclusively the A3AR. It may be suggested that at higher dosages (100 and 1000 μg/kg), receptors other than A3AR were also activated, giving rise to elevation of cAMP production, thereby counteracting the stimulatory effect elicited by A3AR.

Remarkably, potentiation of NK cells upon Cl-IB-MECA treatment was also found in melanoma bearing mice and further confirmation to the capability of Cl-IB-MECA to induce this effect was observed in the adoptive transfer experiments.

IL-12 is defined as an NK stimulatory and cytotoxic factor which exerts a potent anti-tumor effect *in vivo*. It induces INF-γ production by activated T and NK cells and augments cytotoxic activity of these cells via perforin, Fas and TRAIL-dependent mechanisms (26-29). Furthermore, IL-12 has been reported to exhibit anti-angiogenic activity (30). Inhibition of IL-12 production by adenosine and some of its agonists was previously reported. Hasko *et al* showed that adenosine receptor agonists inhibited the production of IL-12 by peritoneal macrophages with the order of potency GCS-21680 (A2AR agonist) > IB-MECA (A3AR agonist) > CCPA (A1AR agonist) (31,32). These results suggest that A2 adenosine receptors have a prominent role in inhibiting IL-12 production by adenosine agonists. It should be noted that the above described effect of IB-MECA was evident at dosages much higher than those used in the study of Hasko *et al* and administered as acute treatment, indicating that IB-MECA's inhibitory effect was mediated via activation of the A2A adenosine receptor. Our results show that oral administration of low-dose Cl-IB-MECA induced increased levels of serum IL-12, which was followed by elevated NK cell activity and tumor growth inhibition.

Activation of the A3AR has been shown previously to elicit direct inhibitory effect on melanoma cell growth *in vitro* (14,16,19,33,34). The A3AR agonist IB-MECA was shown to interfere with the Wnt signaling pathway by decreasing the protein expression level of the effectors PKAc and PKB/Akt, thereby up-regulating GSK-3ß expression level resulting in down-regulation of ß-catenin, cyclin D1 and c-Myc (19). *In vivo*, inhibition of tumor growth by oral treatment with A3AR agonists was demonstrated in melanoma (16), colon (33) and prostate carcinoma (25). Tumor lesions derived from IB-MECA treated melanoma or prostate carcinoma-bearing mice revealed that signaling proteins of the Wnt pathway were modulated in the same manner as was seen *in vitro* (16). Interestingly, the magnitude of the response was greater than that shown *in vitro*. The results of the present study show that an additional indirect mechanism is attributed to the anti-tumor effect of A3AR agonists.

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